5-2024

# Screening full-sib and half-sib families of chestnut seedlings for resistance to Cryphonectria parasitica using a small stem assay and a leaf-disc assay

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Departmental Honors Thesis
The University of Tennessee at Chattanooga
Biology, Geology, and Environmental Science

Examination Date: March 27th, 2024

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## Abstract

*Cryphonectria parasitica* is the ascomycete fungus species that causes chestnut blight disease. Symptoms of chestnut blight include bark cankers and eventually the death of American chestnut, *Castanea dentata*. However, not all *Castanea* species are equally affected by the disease, and some Asian species appear highly resistant. The OALDS and SSA in this study aim to assist in screening for resistance to *C. parasitica* within a year. We investigated the effect of *C. parasitica* on three North American species, one European species, four East Asian species of *Castanea*, and nine full-sib families of TACF backcross hybrids. Our study used both a small stem assay to measure canker lengths and an excised leaf disc assay to measure variation in tolerance to oxalic acid between the different *Castanea* species and hybrids. We inoculated 967 container-grown seedlings with *C. parasitica* strain EP155 and allowed cankers to develop for 12 weeks. Two measurements were recorded for each canker: orange zone and full length of necrosis, following the method of Cipollini et al. 2021. Excised leaf discs were soaked in a 50 mM solution of OA for 8 hours and then digitally imaged for measurement of browning with Image J, following the method of Harden 2023. Results show differences that vary by *Castanea* species in resistance to *C. parasitica*, as measured by orange zone in the SSA was significant between species. OA degradation (browning) did not have any statistically significant results. While the initial results seemed promising, the statistical analysis did not show any significance. These methods may continue to be practiced but should not fully replace the traditional method for *C. parasitica* resistance screening.

## Acknowledgements

Thank you to Dr. Hill Craddock for guidance throughout the thesis process. Thank you to Dr. Jennifer Boyd and Dr. Hope Klug for agreeing to be on my examination committee. Thank you to the Fortwood Greenhouse crew, especially Dr. Paola Zannini. Thank you to Dr. Jared Westbrook and my friend Mert Sekmen for helping me with R. Thank you to the Tucker Foundation for supporting me through scholarships for the last year. Thank you to my friends Ronan Lanam, Joanna Maldanado, Sarah Singleton, Haleigh Sturman, and Rue Wooten for assisting me with hole punching leaves and emotional support throughout this project.

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## Introduction

*Cryphonectria parasitica* is the ascomycete fungus species that causes chestnut blight disease (Roane et al., 1986). Symptoms of chestnut blight include bark cankers and eventually the death of American chestnut, *Castanea dentata*. But not all *Castanea* species are equally affected by the disease, and some Asian species appear highly resistant which could aid in the future of the chestnut breeding program (Craddock & Perkins 2019).

The discovery of chestnut blight caused the United States government to create regulations on bringing fungi and plants across international borders (Roane et al., 1986; Anagnostakis, 1987). The chestnut blight pandemic spread South after being discovered at the Bronx Zoo in New York (Roane et al., 1986). Much of the Eastern United States was dependent on the American chestnut in the early 1900s. As the fungus spread, residents of the Eastern United States lost their livelihoods that depended on the nut production or lumber of the large and then abundant *C. dentata* (Freinkel, 2009; Roane et al., 1986). Hypovirulence and biological control of chestnut blight were some of the first attempts to control the fungus (Griffin, 2000). These methods were successful in Europe; however, we have not seen the same level of success in the United States (Craddock & Perkins 2019). Advocates for *C. dentata* then began breeding for resistance to *C. parasitica* (Burnham, 1987). The breeding program hoped to conserve the chestnut and eventually restore it to its former glory (Westbrook et al., 2019).

Plant breeders working with The American Chestnut Foundation (TACF) are currently testing a small stem assay (SSA) (Cipollini et al., 2021) as a method to screen container-grown *Castanea* seedlings for resistance to chestnut blight disease in a nursery setting (Powell et al., 2007). The SSA method has the potential advantage over the traditional orchard screening in that it can be completed in the first one year of the tree's life, rather than in the five to seven years required to screen field-grown plants (Powell et al., 2007; Cipollini et al., 2021).

Past studies have shown a direct correlation between oxalic acid (OA) and virulence of *C. parasitica* (Bennett & Hindal, 1989). The involvement of oxalic acid in this disease system prompted the initial trial of employing oxalate oxidase (OxO) in genetically modified chestnut trees (Zhang et al., 2013). OxO facilitates the decomposition of oxalic acid into carbon dioxide and hydrogen peroxide (Bolwell & Wojtaszek, 1997). Relative tolerance to OA may provide a new screening option. Oxalic acid tolerance varies greatly between *Castanea* species (Zhang et al., 2013). An oxalic acid leaf disk soak (OALDS) could be a complimentary screening method for blight resistance. This idea was used to look at hybrids in the TACF breeding program (Harden, 2023). In the OALDS, 15 mm disks cut from leaves were soaked in a 50 mM oxalic acid solution and then the proportion of green tissue and browned tissue was measured optically/digitally (Harden, 2023). An effective OADLS method could save time and resources over the SSA.

I conducted both an OALDS and a SSA to compare the two one-growing-season methods and evaluate them as possible proxies for the traditional five- to eight-year orchard-based *Castanea* screening methods. These methods would be incredibly beneficial, if successful, in reducing time and costs of old orchard assays. I predicted that seedlings of *C. dentata* would be the least tolerant to OA due to poor resistance to *C. parasitica*, that seedlings of *C. mollissima* would be the most tolerant to OA due to high resistance to *C. parasitica*, and that the TACF hybrid families would have an intermediate tolerance to OA due to their intermediate blight resistance.

## Hypothesis

I hypothesize *C. dentata* and *C. mollissima* would show significantly different tolerances to oxalic acid, and hybrids would show varying and intermediate levels tolerance to the leaf

soak. Oxalic acid tolerance is expressed in measurable difference in leaf disk necrosis within *C. alabamensis, C. crenata, C. dentata, C. henryi C. mollissima, C. ozarkensis*, *C. sativa, C. seguinii,* and TACF hybrids.

The leaf-disc assay investigates the effect of oxalic acid on different *Castanea* species; it has the potential to characterize species by resistance to blight. The experiment could lead to more accurate breeding for resistance in *Castanea* species and continued conservation of *C. dentata* characteristics. It may also lead to more information on potential breeding between *C. dentata* and other *Castanea* within the same clade for resistance to blight and similar characteristics. We expect to find statistically significant differences between the resistance of *C. dentata, C. dentata* hybrids, and other resistant *Castanea* species. *C. dentata* will be the least resistant to *C. parasitica* and *C. dentata* hybrids will have intermediate resistance. The other *Castanea* species will have more resistance to *C. parasitica,* with *C. mollissima* having the highest resistance.

## Literature Review

### Pre-Blight *Castanea*

The *Castanea* genus belongs to the family Fagaceae. There are ten *Castanea* species in total (Mellano et al. 2018; Mellano et al., 2012; Perkins et al., 2021). The genus is naturally found in Eastern North America, East Asia, Europe, and North Africa (Mellano et al., 2018). The North American species include the American chestnut, *C. dentata*, and the chinquapin species *C. alabamensis, C. ozarkensis,* and *C. pumila* (Mellano et al., 2012; Johnson, 1988). *C. dentata* was very important to animal species in North America because it provided animals with small nutritious chestnuts (Roane et al., 1986). Their six-to-seven-foot diameter trunk and tall slender shape made *C. dentata* a great source of lumber (Brooks, 1937). Historically, *C. dentata* was valued for its fruit eaten often by the people of Appalachia (Freinkel, 2009). The wood of *C. dentata* is very high in tannins and was used to dye leather and aided in outdoor use of the lumber (Freinkel, 2009; Roane et al., 1986). *C. dentata* was very useful as a crop and was gathered, eaten, sold, and fed to livestock (Freinkel, 2009; Roane et al., 1986). In certain North American regions, as much as 25% of the forest canopy was occupied by *C. dentata*, offering shade to the floor of the forest (Roane et al., 1986).

There is only one European species, *Castanea sativa* Mill; it is the only native chestnut in Europe (Conedera et al., 2016; Lang et al., 2006). The *Castanea* species in Europe are concentrated in Italy, France, Spain, and the Iberian Peninsula (Conedera et al., 2016). *C. sativa* trees are valued for their timber and sweet chestnut fruit (Lang et al., 2006).

*Castanea* is found in East Asia, where it is thought that chestnut blight originated (Anagnostakis and Hillman, 1992). Asian species include *C. mollissima, C. henryi, C. seguinii,* and *C. crenata* (Mellano et al., 2012).

**Chestnut Blight**

*Cryphonectria parasitica*, an ascomycete fungus, was first observed on *C. dentata* at the Zoological Park of New York City in 1904 (Roane et al., 1986). The fungus is identified by necrotic lesions appearing on limbs and the trunk, accompanied by orange spore formation that progresses into lethal cankers. (Murrill, 1906; Rankin, 1912). Chestnut blight was accidentally introduced to the United States from Japan in *C. crenata* nursery stock in the early 20th century (Anagnostakis & Hillman, 1992). Within forty years the disease spread all over the natural range of *C. dentata*. The fungus killed an estimated four-billion American chestnut trees (Roane, 1986). *C. parasitica* is a necrotrophic fungus. Necrotrophic fungi kill their hosts for increased survival instead of keeping the host alive like a typical parasite.

The highest resistance to blight is found in the native Asian *Castanea* species, namely the Chinese chestnut (*C. mollissima*) and the Japanese chestnut (*C. crenata*). The European chestnut (*C. sativa*) exhibits less susceptibility to blight compared to the American chestnut but is more susceptible than the resistance seen in the Asian species (Nguyen 2023).

*Cryphonectria parasitica* infects trees through sexual and asexual spores. The fungus enters wounds in *Castanea* and breaks down the vascular cambium, hindering tree growth (Rigling & Prospero, 2018). Infected trees have lesions with reddish-brown discoloration on the bark at the infection site (Rigling & Prospero, 2018). *C. parasitica* entry is often initiated by growth cracks, cuts, fires, or drought (Rigling & Prospero, 2018).

The American Chestnut Foundation breeds *C. dentata.* The original thought to backcross *C. dentata* and *C. mollissima* for resistance was suggested by Burnham (1987). Through a process of backcrossing and careful screening for blight resistance in each generation, Burnham (1987) aimed to diminish the characteristics of *C. mollissima* (diluted by half every backcross

generation) while moving the Asian blight resistance alleles into *C. dentata*. Unfortunately, introgression of blight resistance into *C. dentata* has not been that simple (Craddock and Perkins 2019).

**Hypovirulence**

Transmissible hypovirulence in *C. parasitica* was first discovered in Italy by a French plant pathologist, Jean Grente, who coined the term and hypothesized that a virus was responsible for the phenomenon (Roan et al., 1986; Anagnostakis & Hillman, 1992). In Europe, *C. sativa* was affected, and hypoviruses successfully controlled the pathogen, reducing its harm to chestnut trees (Anagnostakis & Hillman, 1992). The hypovirus *Cryphonectria hypovirus-1* hinders fungal sporulation, primarily spreading through hyphal anastomosis. It aids in American chestnut conservation by promoting canker formation at infection sites and limiting the spread of ascospores (Chen & Nuss, 1999). These strains contribute to biocontrol efforts by weakening the fungus and facilitating tree recovery while limiting spore dispersion.

**Oxalic Acid**

Some pathogenic fungi employ oxalic acid to weaken cell walls (Rigling & Prospero, 2018). It has been demonstrated that oxalic acid acidifies host tissue and sequesters calcium from the host cell walls (Dutton & Evans, 1996). This causes a toxic effect on the host cells leading to cell wall degradation (Havir & Anagnostakis, 1983). There is a direct correlation between virulence factors and oxalic acid production (Dutton & Evans, 1996). *C. parasitica* secretes oxalic acid at the infection's advancing edge, causing damage to *Castanea* (Bennett & Hindal, 1989).

A higher virulence level of *C. parasitica* may indicate increased oxalic acid production, which has been considered as a stand in for the more intensive SSA, though past attempts have

not been successful (Harden, 2023). *C. parasitica* utilizes oxalic acid to attack vulnerable trees (Zhang et al., 2013). In infection, *C. parasitica* excretes oxalic acid as oxalate into the chestnut tree's stem, lowering the pH and causing localized damage (Zhang et al., 2013).

Some studies suggest that oxalic acid may induce apoptosis (programmed cell death), facilitating the necrotrophic fungus (Errakhi et al., 2008; Rigling & Prospero, 2018). When the *Castanea* stem is vulnerable, fungal hyphae enter through the cambium which causes the distinctive canker formation (Griffin, 2011). Researchers may be able to inhibit the oxalic acid pathway. Removing the ability to produce oxaloacetate acetyl hydrolase in *C. parasitica* reduces the fungus's ability to form cankers (Chen et al., 2010). In the metabolic pathway, oxaloacetate acetyl hydrolase catalyzes the hydrolysis of oxaloacetate to oxalic acid and acetate (Chen et al., 2010). This could be beneficial in future studies aimed at *Castanea* restoration in North America.

**Chestnut Breeding**

American chestnut breeding efforts began after blight first arrived in North America in the 1950s (Clapper, 1954; Graves, 1950). Some successful early Backcross 1 (B1) trees were named after Arthur Harmount Graves and Russell B. Clapper because of their contributions to breeding efforts (Steiner et al., 2017). Some of these trees are still used in the TACF breeding program (Hebard et al., 2012). The idea of backcross breeding was proposed by two scientists, Phillip A. Rutter and Charles R. Burnham (Burnham, 1988). The purpose of backcross breeding is to introgress alleles from the resistant *C. mollissima* into the susceptible *C. dentata* (Burnham, 1988). Backcross breeding must also retain the adaptive and characteristic traits of *C. dentata* (Burnham, 1988). Burnham held the belief that blight resistance exhibited partial dominance (Hebard et al., 2012).

The F1 generation is intermediately resistant to blight (Powell et al., 2019). When F1 generations are crossed with *C. dentata*, half of the traits from *C. mollissima* are lost in the resulting first backcross generation (Fernandes et al., 2022). A cross between an F1 tree and *C. dentata* produces the BC1 or B1 generation (Hebard et al., 2012). The following cross of B1 crossed with *C. dentata* produces BC2 or B2 generation (Hebard et al., 2012). Following three backcrosses with the recurrent species *C. dentata*, just one-sixteenth of the genetic material from *C. mollissima* will remain. Burnham predicted that recovery of full, Chinese levels of resistance would be possible by intercrossing selected B3s in a B3F2 generation, and that resistance would be fixed (homozygous at all loci) in the B3F3s (Burnham, 1988).

After creating many generations of hybrid trees including B3F2s and B3F3s over the past 40 years, genetic studies reveal that Burnham's hypothesis cannot be supported, suggesting that the envisioned backcross breeding by Burnham and Rutter may not be feasible (Miller, 2020). Unfortunately, recent genetic studies have shown that blight resistance in *C. mollissima* may be controlled by dozens of genes scattered among all twelve chromosomes (Westbrook et al., 2020). Although backcross breeding has not been as successful as previously hoped, there is still conservation value in the surviving hybrids (Westbrook et al., 2020).

Currently TACF is the major organization involved in *Castanea* conservation efforts (*The American Chestnut Foundation*, n.d.). The main goal of conservation of *Castanea* and resistance to *C. parasitica* is to retain as much genetic material from *C. dentata* as possible (Westbrook et al., 2019). It is also important to retain the defining characteristics of *C. dentata* (Westbrook et al., 2019). Hybrids accumulated over the last thirty years will continue to be used in the breeding effort by producing seedlings for the best x best experiments.

**Transgenic Chestnut Trees**

Genetic engineering is a new technology used to assist scientists; especially as local environments are subject to change. No species of *Castanea* have the natural genetic ability to metabolize oxalic acid (Carlson et al., 2022). Using plants like wheat (contains the OxO gene), which metabolizes oxalic acid, researchers began investigating the possibility of genetically engineering the American chestnut (Carlson et al., 2022; Zhang et al., 2013). In the presence of oxalic acid, the wildtype tissues demonstrated a significant decrease in lignin content and increase in cellulose content while transformed tissue did not. Transforming chestnut may increase resistance to *C. parasitica* because it may limit changes in the cell wall composition caused by the fungus (Welch et al., 2007). In theory, using the OxO gene from wheat plants, *Castanea* can break down OA when attacked by *C. parasitica* (Bolwell & Wojtazek, 1997). Agrobacterium-mediated transformation was inserted into *C. dentata* by scientists at The State University of New York College of Environmental Science and Forestry (Westbrook et al., 2020). This would elevate the ability of *C. dentata* to fight *C. parasitica* (Carlson et al., 2022; Powell et al., 2019). The genetically engineered trees from SUNY-ESF showed a higher resistance to *C. parasitica* in early trials (Steiner et al., 2017).

The Darling 58 (D58) is genetically transformed *C. dentata* (Westbrook et al., 2020). After much research and time put into developing Darling 58, TACF has decided to remove support from D58. TACF reported disappointing and variable results throughout its chapters (Fitzsimmons et al., 2023). TACF reports increased leaf browning and mortality in D58 (Fitzsimmons et al., 2023). These are disappointing results but perhaps research into transgenic chestnut trees will continue.

## Screening Methods for Blight

Before the adoption of modern SSAs, the earlier screening technique involved assessing trees already planted in orchards (Powell et al., 2007). Typically, trees would grow for four to five years until their trunk diameter reached 2.5 to 5 cm, making them suitable for evaluation (Griffin et al., 1983). A 5 mm hole was created to insert pathogen-infected agar into the tree using the cork-borer method (Griffin et al., 1983). Subsequently, the trees were infected with fungus and many of them died. The trees that remained could be advanced to the next generation in the backcross program. The traditional screening method is beneficial because it can identify intermediate or partial resistance to *C. parasitica* in hybrid trees (Anagnostakis & Hillman, 1992). This screening, with its high resolution, enables selections within backcross families. However, since all trees are intentionally infected with blight, most of them eventually succumb to the disease. Over the five to eight years required to complete the screening, substantial resources are invested in caring for the seedlings. In an effort to reduce costs, time, and resources associated with proper tree screening, the SSA was introduced (Cipollini et al., 2021).

## Small Stem Assays

Small stem assays serve as a nursery screening method for container-grown seedlings, implemented to assess trees before orchard planting and to conserve resources (Powell et al., 2007). Originating in 1989, Herbard and Shain utilized SSA by inoculating five to eighteen-month-old seedlings to test for blight resistance. This revealed significant differences between *C. dentata* and *C. mollissima* but had limited success in assessing hybrid resistance (Hebard & Shain, 1989). In 2017, TACF collaborated with the U.S. Forest Service to enhance the SSA (Westbrook, 2018).

In the following years Cipollini et al. (2021) proposed an improved SSA, inoculating cut stems at the tip instead of the side, addressing previous issues and providing consistent inoculation results. Seedlings screened with the cut stem method exhibited better out-planting survival rates than those inoculated with the cork-borer method (Cipollini et al., 2021). Unlike orchard assays requiring 5-year-old trees, SSA can be conducted in a blight-free greenhouse setting with one to two-year-old seedlings, proving effective in differentiating resistance levels (Westbrook & Jarret, 2018). Notably, differences in blight resistance were discerned in 68 families of BC3F2 at the seedling stage. This was particularly evident when inoculated with highly pathogenic *C. parasitica* strains (Westbrook & Jarret, 2018).

For the present work, I investigated the effect of *C. parasitica,* on three North American species, one European species, four east Asian species of *Castanea*, and nine full-sib families of TACF backcross hybrids. My study used both a small stem assay to measure canker lengths and an excised leaf disc assay to measure variation in tolerance to oxalic acid between the different *Castanea* species and hybrids.

## Materials and Methods

### Planting

The seeds I used for the small stem assay (SSA) and oxalic acid leaf disk soak (OALDS) are listed in Table 1. Dr. Craddock and Dr. Zannini stratified seeds in the UTC Fortwood Greenhouse walk-in cooler at 40F from October 2022 until sowing. The seeds were given to us by colleagues. The *C. alabamensis* came from the Cochran Backcross Orchard in Moore County, Tennessee. The seeds of *C. crenata* and *C. dentata* came from Sara Fitzsimmons and Steve Hoy at the Pennsylvania State University. Dr. Marty Cipollini at Berry College provided seeds of *C. henryi, C. mollissima* GAFL1, and *C. seguinii.* Dr. Craddock obtained *C. mollissima* AU from

Camp Hill Alabama orchard. Dr. Ron Revord at the University of Missouri provided *C. ozarkensis*. The *C. sativa* was purchased by Dr. Jared Westbrook from a commercial source. The *C. sativa* were grown in Italy. The Dave Cantrell Orchard in Corryton Tennessee provided the *C. henryi* DC 13-3-13. All the crosses came from the Tennessee chapter of the American Chestnut Foundation. The crosses are the progeny of selected trees in the American Chestnut Foundation breeding program. The seeds were planted by Fortwood Greenhouse Crew from early February to late March 2023 in CP512 pots, which are five-inch by twelve-inch square pots which hold five liters. We planted the seeds in Pro-Mix BK55, a potting medium composed of 25-35% sphagnum peat moss, 55% processed southern pine bark, perlite, limestone, and a wetting agent. Pro-Mix BK55 was top-dressed with a tablespoon of Osmocote Plus, a slow release, long-lasting, and nutrient rich fertilizer that aids in promoting healthy plant growth. Osmocote Plus provides the seeds with micronutrients over a six- to eight-month period in the nursery. We moved the trees outside to the nursery when the weather settled in May 2023. Many of the trees were staked for vertical growth with bamboo poles.

**Greenhouse Management**

The trees were grown in full sun and watered using a drip irrigation line. The trees were watered as needed and re-staked, if necessary, throughout the growth period. They were observed and cared for by members of the Fortwood Greenhouse Crew.

**Experimental Design**

We set the trees up in a completely randomized design along ten adjacent rows in the nursery (similar to the method of Nguyen (2023) -- who used non-adjacent blocks in a randomized complete block design -- but because in 2023 the nursery was too full; we used a completely randomized design with adjacent blocks). The trees' positions in the nursery were

assigned by labelling each tree with a unique number generated by a random number generator in Excel.

**Preliminary Tests**

The oxalic acid assay leaf disk soak is a relatively new experiment, so three preliminary tests had to be done to set the parameters for the larger experiment. The first test was to demonstrate that the percent necrosis of leaf disks from the control species could be separated statistically; the second trial was to determine optimal soak time; and the third was to show the importance of standardizing leaves so that comparisons could be made between plants.

The first preliminary trial was designed to measure the difference between *C. dentata* and *C. mollissima* in their response to an OA leaf disk soak. The third fully expanded leaf from the shoot tip was chosen based on previous studies (Harden, 2023). There was a significant difference in area (percentage) leaf necrosis between species in reaction to an oxalic acid.

In previous experiments (Harden, 2023) soaking time in OA solutions ranged from eight to 12 hours. The leaf soaks I preformed soaked for eight hours before the American leaves died (turned completely brown). When the leaves die, they are no longer usable in determining resistance to oxalate acid. The leaves must be removed from the acid before complete browning occurs. The leaves were checked every hour for nine hours and removed from the acid after nine hours. However, most of the *C. dentata* controls turned completely brown by the end of the nine-hour period, but eight hours seemed to be the threshold for most *C. dentata* and hybrids.

The third preliminary test determined if leaves reacted differently to oxalic acid based on its position on the tree. I selected the third, fifth, and seventh fully developed leaf from the tip of the growing shoot. I also selected the leaf eighteen inches from the soil (measuring upward). Some of the leaf selections overlapped so the third leaf and the leaf eighteen inches from the soil

were the same. The third leaf and the leaf eighteen inches from the ground had the same statistical value, so for the rest of the leaf disks I used leaf three.

**Oxalic Acid Leaf Soak**

I hypothesize *C. dentata* and *C. mollissima* would show significantly different tolerances to oxalic acid, and hybrids would show varying and intermediate levels tolerance to the leaf soak. I used the same nineteen half-sib families of open-pollinated seedlings in my oxalic acid leaf disk assay as the SSA. The *C. dentata* family came from Connecticut. The control *C. mollissima* family came from an orchard located at Auburn University (Table 1).

**Table 1.**

*Family, quantity, and seed type for seedlings used in the 2023 SSA and OALDS experiments.*

| Family | Quantity | Seed type | Source |
|---|---|---|---|
| *C. alabamensis* | 46 | Al | Cochran Backcross Orchard, Moore County, TN |
| *C. crenata* | 87 | Jp | Sara Fitzsimmons and Steve Hoy at Penn State |
| *C. dentata* | 131 | Am | Sara Fitzsimmons and Steve Hoy at Penn State |
| *C. henryi* | 106 | He | Dr. Marty Cipollini at Berry College |
| *C. henryi* DC13-3-13 | 22 | He | Dave Cantrell Orchard, Corryton, Knox County, TN |
| *C. mollissima* AU | 100 | Ch | Drs Hill and Paola Craddock from Camp Hill Alabama Orchard at Auburn University |
| *C. mollissima* (GAFL1) | 19 | Ch | Dr. Marty Cipollini at Berry College |
| *C. ozarkensis* | 100 | Oz | Dr. Ron Revord at the University of Missouri |
| *C. sativa* | 53 | Eu | Jared Westbrook purchased at a commercial store (from Italy) |
| *C. seguinii* | 67 | Seg | Dr. Marty Cipollini at Berry College |
| CCCG-61 x GABE001-165 | 41 | F2 | TN Chapter of the American Chestnut Foundation |
| CCCG-61 x GABE001-297 | 55 | BB1 | TN Chapter of the American Chestnut Foundation |
| CCCG-78 x GABE001-165 | 28 | BB1 | TN Chapter of the American Chestnut Foundation |

| | | | |
|---|---|---|---|
| CCSP-13-140 x CCSP-7-134 | 15 | BB1 | TN Chapter of the American Chestnut Foundation |
| CCSP-13-140 x TNRC09-3-9 | 34 | BB1 | TN Chapter of the American Chestnut Foundation |
| CCSP-1-79 x CCSP-3-50 | 18 | BB1 | TN Chapter of the American Chestnut Foundation |
| CCSP-1-79 x CCSP-B2-3/7 | 15 | BB1 | TN Chapter of the American Chestnut Foundation |
| TN-RC09-2-22 x GAHR001-D39 | 13 | B4F2 | TN Chapter of the American Chestnut Foundation |
| TN-RC09-6-46 x GABE001-297 | 18 | B4F2 | TN Chapter of the American Chestnut Foundation |

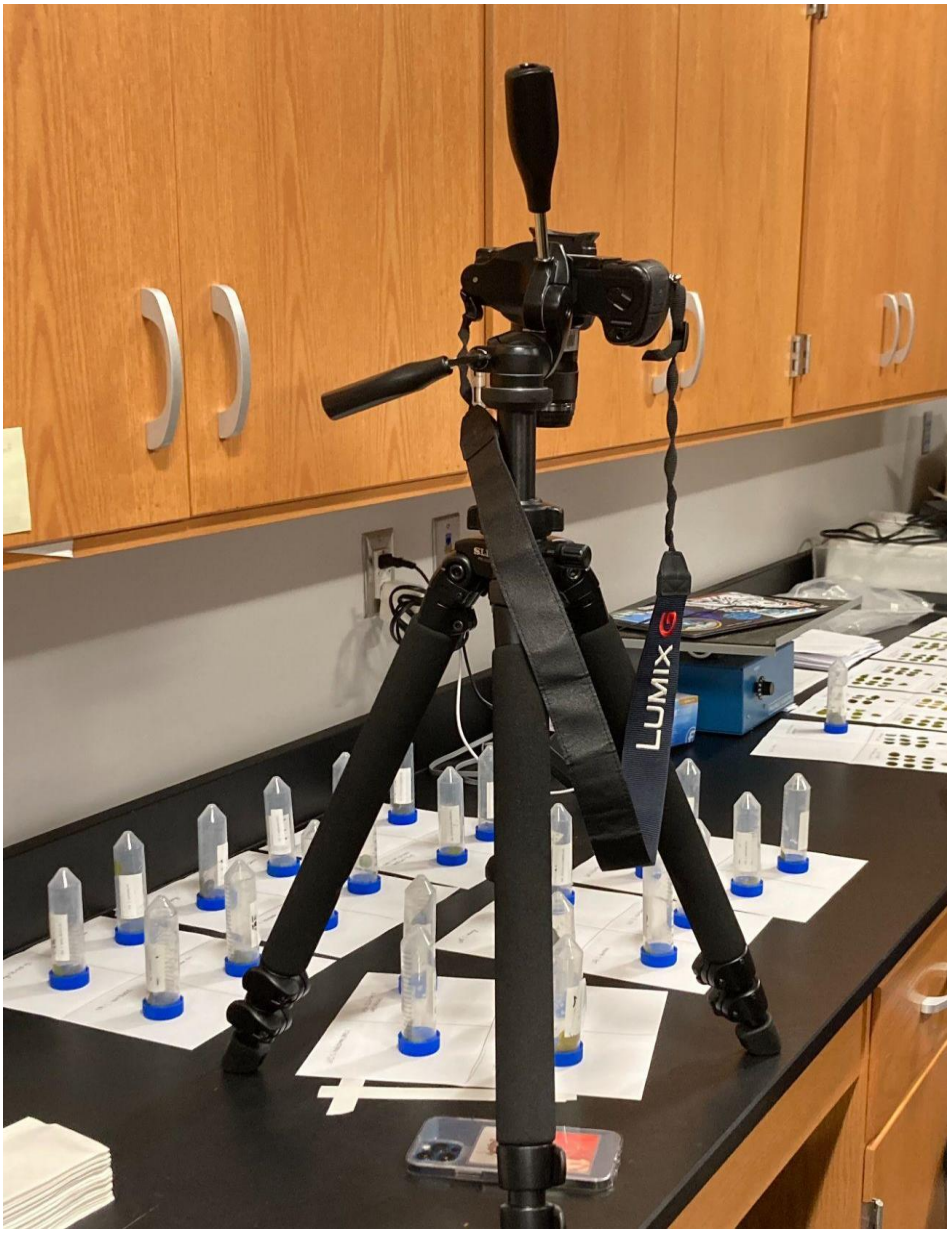
Al = *C. alabamensis* x Open Pollinated (OP); Jp = *C. crenata* x OP; Am = *C. dentata* x OP; He = *C. henryi* x OP; Ch = *C. mollissima* x OP; Oz = *C. ozarkensis* x OP; Eu = *C. sativa* x OP; Seg = *C. seguinii* x OP; F2 = Progeny of First Generation; BB1 = F1 x Selected Backcross Tree; B4F2 = BC4 x BC4

The nineteen half-sib families in the Oxalic Acid Leaf Disk Soak Assay are listed in Table 1 along with the quantity, type, and source of the seeds. One-hundred and forty-five trees of the total nine-hundred and sixty-eight container grown seedlings were used in this study. I used three adjacent leaves per tree to make a total of ten disks (three to four disks per leaf). This means every tree had ten disks each. There were a total of 1450 disks in the assay. Once the leaves were cut, I put all the disks from one tree in a 50mL container. I repeated this for the leaf disks for each tree. The containers were labeled with the tree's number and species. I soaked the leaves for eight hours in the 50mMol oxalic acid solution. I made the solution in the lab using one liter of deionized water for every 4.50 grams of anhydrous oxalic acid. I used a simple scale and a weigh boat to measure the acid when I made the solution. I filled all the containers with 30 L of 50mMol solution. I left the leaf disks soaking for eight hours on a shaker.

After eight hours I removed the leaves from the solution using a small strainer. I then placed all the leaves from one tree on a piece of paper labeled with the tree number and species. I took photographs of all ten disks from each tree and analyzed those images.

**Figure 1:**

*Oxalic acid soak methods and camera setup*



**SSA Inoculum**

Cultures of SG2-3, Weekly, and Ep155 strains of *C. parasitica* were shipped from the TACF Research Farms in Meadowview Virginia. The colonies plated on June 21st, 2023, were refrigerated from June 23, 2023, to July 5th, 2023; colonies plated on June 29th, 2023 were refrigerated from July 3rd to July 5th 2023. The cultures were set under a window on a lab bench at room temperature one week before the inoculation date to promote growth of the fungus.

**Inoculation**

By July 17th, 2023, the trees had ample time to grow to a 5mm diameter. Of the 968, only 820 were large enough to be inoculated. The trees that did not reach the 5 mm diameter requirement were logged and removed from the study. Also, atypical looking trees were excluded from the experiment. Using the uniform 5 mm and 5.5 mmm cutout, the branch that had the longest internode and a 5 mm diameter was snipped off using pliers (Cipollini et al., 2021). Mycelium-containing agar was extracted from the petri dish using a 5 mm straw. A flame sterilized spatula was also used to assist in extracting mycelium containing agar. The mycelium-containing agar was placed in an approximately 50 mm long straw tip with one sealed end. The mycelium side of the agar was placed on top of the tree over the wound while still inside the straw tips. The straw tips used to cover the tree wounds were cleaned by soaking them in bleach.

Plastic straw tips were used to create a waterproof seal over the inoculum. The straw is approximately 5 mm in diameter which fits over the wound of the tree. There were also 5.5 mm diameter straws for the larger specimens. The straw tips covered the tree wounds for 1 week because the tips created a waterproof seal that protected the inoculum. The straws were cut into 50 mm lengths, and one end was sealed at the 40 mm mark with a FoodSaver vacuum sealer.

After the individual was inoculated it was returned as close to the original row as possible with the volume of trees inoculated. The extra mycelium plates were returned to the lab refrigerator for storage.

**Figure 2:**

*Orange zone versus full necrosis zone on* C. dentata.

**Data Collection and Screening**

ImageJ is an open-source Java-based image editing program (Rasband, 1997-2018). Used in many biological sciences, ImageJ can be used to measure objects in images (Abrámoff et al., 2004). In this experiment, I used it to measure individual leaf disks, then measure the remaining green area following the methods of Harden 2023. After soaking in acid, the leaves were rinsed in distilled water and photographed using a tripod, a Lumix GH4 Hybrid Camera, and an Olympus MSC ED M 60mm Macro Lens. All ten disks were photographed at once, so only one photograph was taken per tree.

The trees were monitored, and any abnormalities were noted. At 90 days post inoculation, on October 9th, 2023, the orange zone and the canker were measured on each tree. The orange zone was measured at the furthest point of orange of color change seen on the tree within the canker area. For consistency, the same person measured every single tree.

**Analysis**

RStudio is an open-source software for statistical analysis and graphic development (RStudio, 2019). Ggplot was used specifically for developing visually appealing images (Wickham, 2016). Data were analyzed using RStudio (Team, 2023) and were subjected to a one-way ANOVA to determine the effect of the inoculum. ANOVA was performed on each family and generation. Differences were considered statistically significant at $p \leq 0.05$. I sent my SSA data to Dr. Jared Westbrook at the American Chestnut Foundation. He provided me with a correlation coefficient using global data, and a Duncan's multiple range test (Figure 3, 4, 5, 6.)

## Results

**One Way ANOVA**

The data comparing non-hybrid families and orange zones passed the Shapiro-Wilk Normality Test with a p-value < 0.001 (Table 6). The data comparing orange zones and all families also passed with a p-value of < 0.001 (Table 8). The data collected did not pass the Grubb's test for outliers. Tree 312 *C. segunii* was removed from the data as its orange zone length was 410 mm. The remainder of the data was not significant.

**Table 2.**

*ANOVA table of OA necrosis to non-hybrid families.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Tree** | 9 | 768 | 85.29 | 1.363 | 0.215 |
| **Residuals** | 99 | 6193 | 62.55 | | |

**Table 3.**

*ANOVA table of OA necrosis between hybrid families.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Tree** | 8 | 331.9 | 41.48 | 0.453 | 0.878 |
| **Residuals** | 27 | 2473.7 | 91.62 | | |

**Table 4.**

*ANOVA table of OA necrosis between all families.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Tree** | 18 | 1302 | 72.33 | 1.052 | 0.409 |
| **Residuals** | 126 | 8666 | 68.78 | | |

**Table 5.**

*ANOVA table of OA necrosis between clades.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Clade** | 2 | 207 | 103.50 | 1.506 | 0.225 |
| **Residuals** | 142 | 9761 | 68.74 | | |

**Table 6.**

*ANOVA table of orange zone between non-hybrid families.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Cross** | 9 | 62388 | 6932 | 13.4 | <2e-16 |
| **Residuals** | 607 | 314104 | 517 | | |

**Table 7.**

*ANOVA of orange zone between hybrid families.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Cross** | 8 | 3845 | 480.6 | 1.404 | 0.197 |
| **Residuals** | 192 | 65741 | 342.4 | | |

**Table 8.**

*ANOVA of orange zone between all families.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **All Trees** | 18 | 66239 | 3680 | 7.741 | <2e-16 |
| **Residuals** | 799 | 379845 | 475 | | |

**Table 9.**

*ANOVA of orange zone between clades.*

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr (>F)** |
|---|---|---|---|---|---|
| **Clade** | 2 | 977 | 488.7 | 0.895 | 0.409 |
| **Residuals** | 815 | 445107 | 546.1 | | |

**Tukey's HSD and Duncan's Multiple Range Test**

Tukey's HSD test was performed post-hoc on the significant ANOVAs.

**Table 10.**

*Tukey's HSD adjusted p-value of orange zone between non-hybrid families.*

| **Contrast** | **Adj. p. Value** |
|---|---|
| *C. dentata – C. alabamensis* | 1.492601e-02 |
| *C. henryi – C. alabamensis* | 1.662951e-05 |
| *C. ozarkensis – C. dentata* | 1.063567e-05 |
| *C. crenata – C. dentata* | 1.214569e-02 |
| *C. henryi DC 13-3-13 – C. dentata* | 1.507055e-02 |
| *C. mollissima* AU *– C. dentata* | 8.266029e-10 |
| *C. mollissima* GAFL1 *– C. dentata* | 4.649204e-04 |
| *C. sativa – C. dentata* | 3.463861e-02 |
| *C. henryi – C. ozarkensis* | 5.942027e-10 |
| *C. henryi – C. crenata* | 2.386926e-06 |
| *C. henryi* DC 13-3-13 *– C. henryi* | 6.795490e-05 |
| *C. mollissima* AU *– C. henryi* | 3.939687e-10 |
| *C. mollissima* GAFL1 *– C. henryi* | 1.026095e-06 |
| *C. sativa – C. henryi* | 3.214008e-05 |
| *C. seguinii – C. henryi* | 1.238281e-02 |

**Table 11.**

*Tukey's HSD adjusted p-value of orange zone between all families.*

| **Contrast** | **Adj. p. Value** |
|---|---|
| *C. dentata* – *C. alabamensis* | 2.775643e-02 |
| *C. henryi* – *C. alabamensis* | 1.821167e-05 |
| *C. ozarkensis* – *C. dentata* | 1.117648e-05 |
| *C. crenata* – *C. dentata* | 1.236247e-02 |
| *C. henryi* DC 13-3-13 – *C. dentata* | 2.803767e-02 |
| *C. mollissima* AU – *C. dentata* | 0.000000e+00 |
| *C. mollissima* GAFL1 – *C. dentata* | 6.815994e-04 |
| *C. henryi* – *C. ozarkensis* | 0.000000e+00 |
| *C. henryi* – *C. crenata* | 2.176071e-06 |
| *C. henryi* DC 13-3-13 – *C. henryi* | 8.450413e-05 |
| *C. mollissima* AU – *C. henryi* | 0.000000e+00 |
| *C. mollissima* GAFL1 – *C. henryi* | 8.589797e-07 |
| *C. sativa* – *C. henryi* | 3.737630e-05 |
| *C. seguinii* – *C. henryi* | 2.282117e-02 |
| CCCG-61 x GABE001-165 – *C. henryi* | 3.582274e-03 |
| CCCG-61 x GABE001-297 – *C. henryi* | 7.329711e-05 |
| CCSP-13-140 x TNRC09-3-9 – *C. henryi* | 2.174543e04 |

**Figure 3.**

*Histogram of 90-day Canker Rating Separated by Cross*

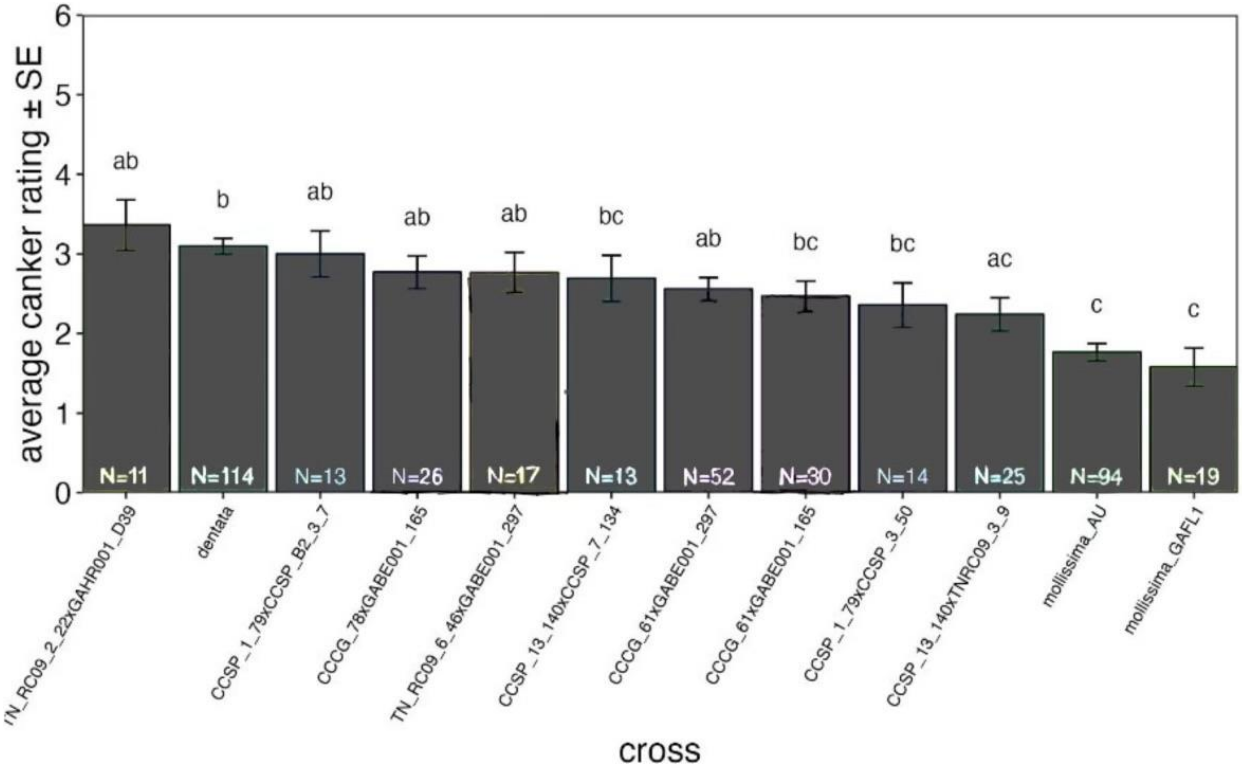
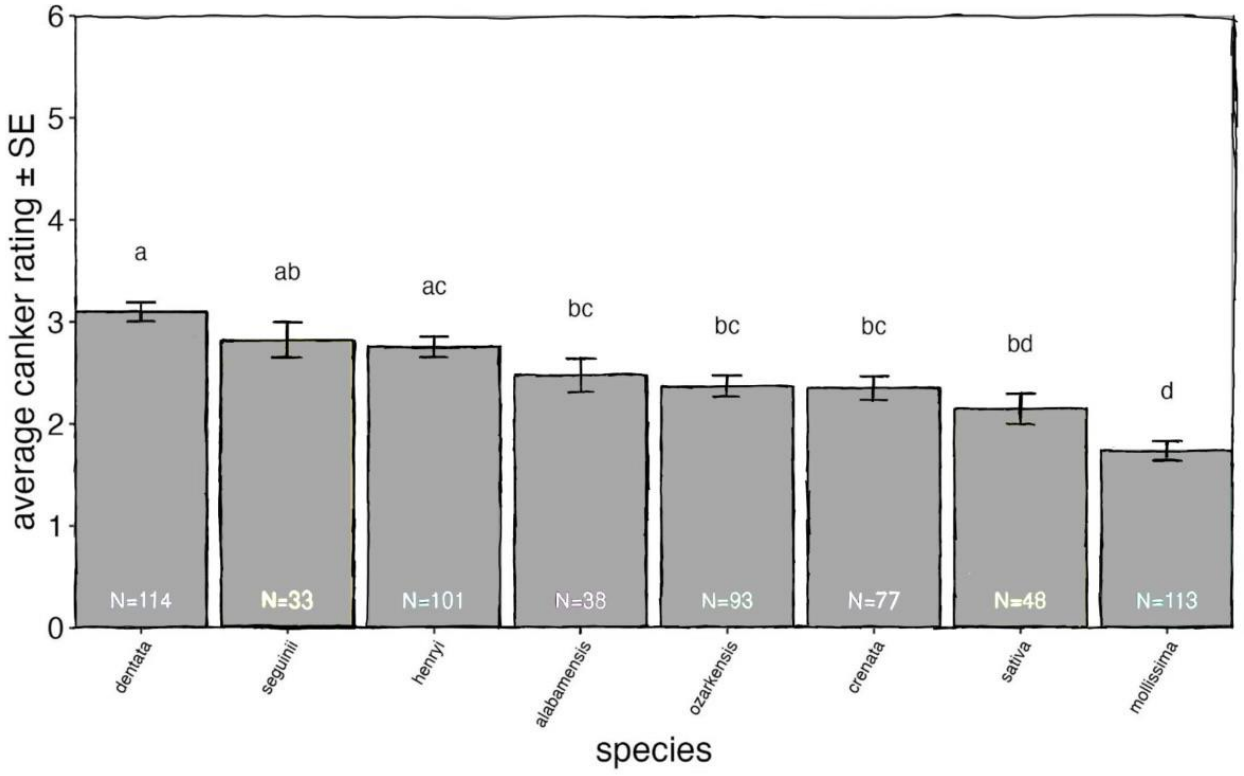


**Figure 4.**

*Histogram of 90-day Canker Rating Separated by Species*




*Note.* Values having a common letter are not statistically significantly different ($p<0.05$) as determined by Duncan's multiple range test. Performed by Dr. Wesbrook.

## Mother Ancestry Correlations

When examining the correlation between *C. dentata* ancestry and the average blight resistance of the offspring, a negative correlation with an r-value of -0.90 was observed. Most families have over 0.5 mother *C. dentata* ancestry but have varying resistance levels.

In a similar analysis correlating *C. dentata* ancestry with blight resistance using the scaled orange zone, the resulting r-value is 0.92. Varying levels of blight resistance are observed. Dr. Westbrook provided me with a correlation coefficient using global data.

**Figure 5.**

*Correlation Between the Mid Parent Blight Resistance Index and Progeny Mean Blight Resistance*

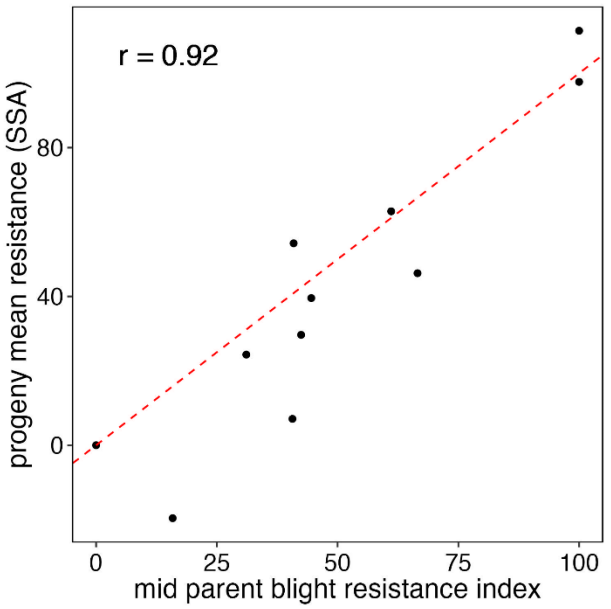
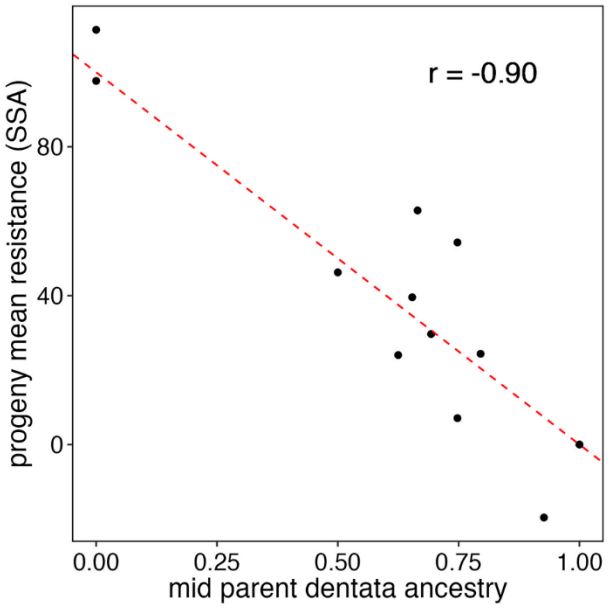



**Figure 6.**

*Correlation Between the Amount of* C. dentata *Ancestry and Progeny Mean Blight Resistance*



## OA Necrosis and Average Canker Length

*C. dentata* and *C. mollissima* showed statistically significant differences when comparing their orange zones. The average OA necrosis separated by crosses shows the lack of significant statistical differences. Most of the averages are very similar throughout the clades.

**Figure 7.**

*Average percentage of OA necrosis separated by species and crosses.*

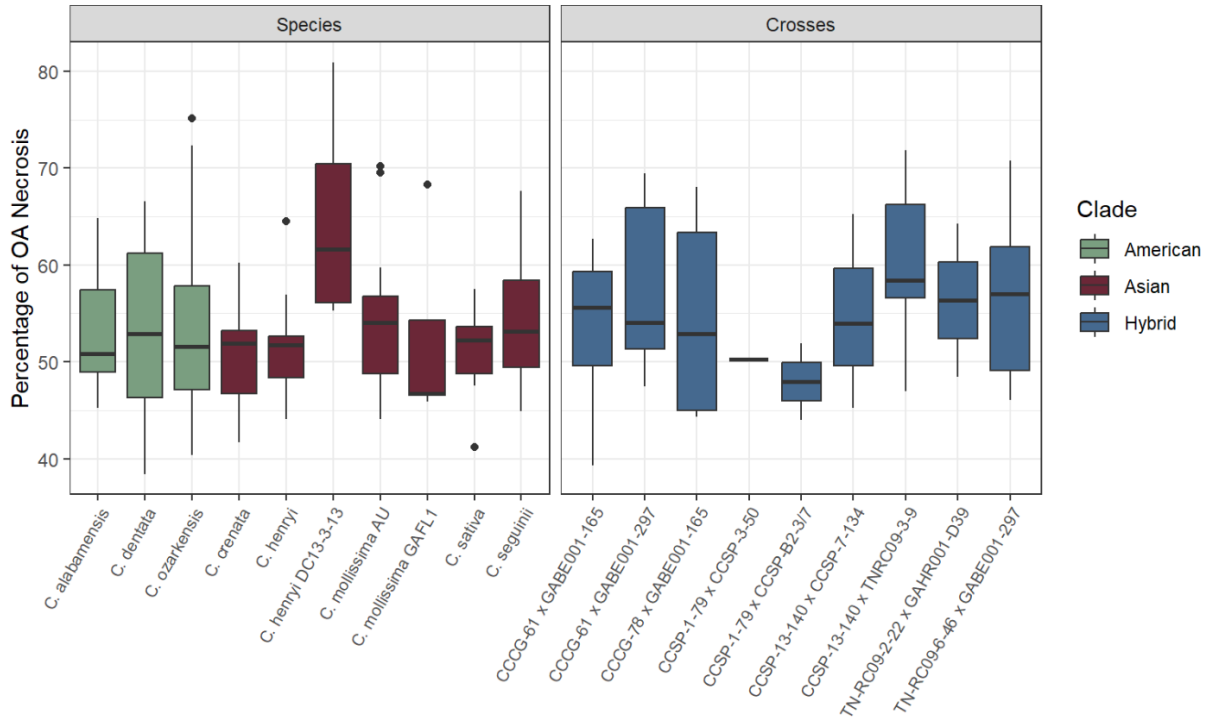
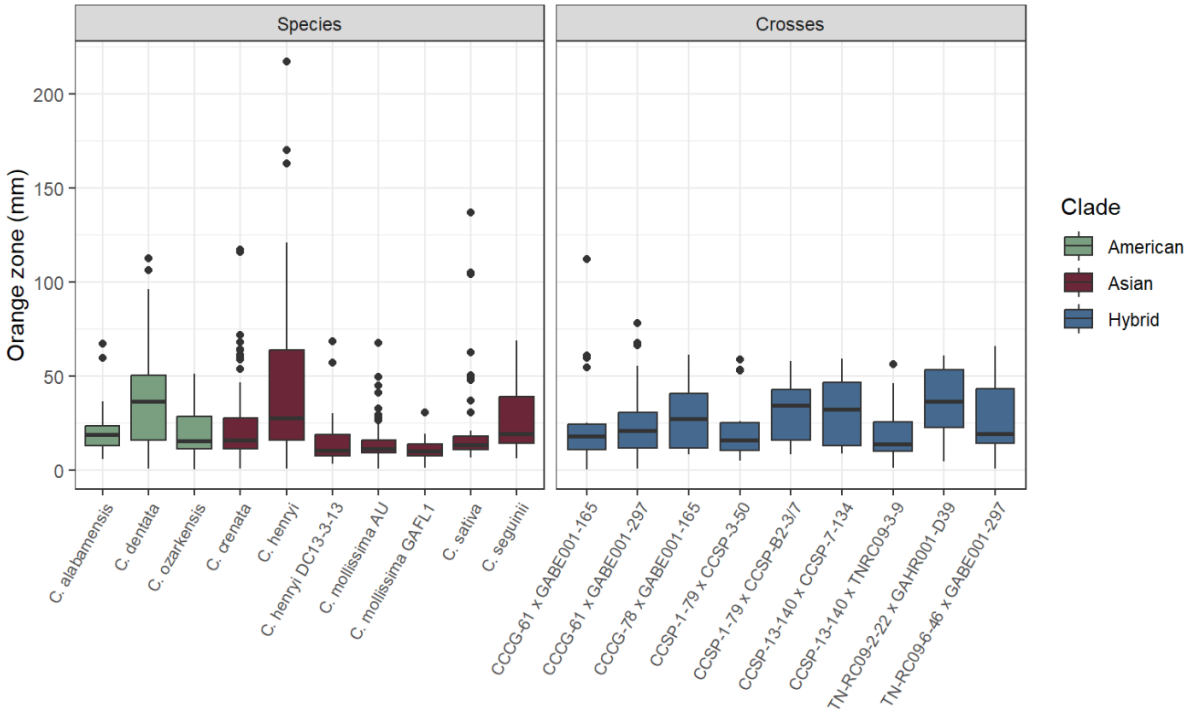


**Figure 8.**

*Average length of orange zone separated by species and crosses.*




*Note.* Clades were determined by most closely related species. All crosses were given a separate clade for best comparison.

## Discussion

### Average Canker Lengths

*Castanea dentata* exhibited the highest average canker lengths among all tested families, while *C. mollissima* displayed the lowest mean compared to others. Hybrid generations showed canker length means between the two controls. ANOVA analyzed mean differences for each family, yielding statistically significant differences between *C. dentata* and *C. mollissima* ($p<0.001$). However, discerning differences among hybrids was challenging as it has been in past experiments. Some hybrid families resembled *C. mollissima*, with a distinct mean, while others

resembled *C. dentata* but lacked statistical significance. My SSA results agree with those of Nguyen 2023 even without implementing the block design. This highlights why the SSA might not be suitable for artificial selection within generations or families due to its limited resolution in predicting blight resistance.

In Figures 7 (OALDS results) and 8 (SSA canker lengths) there is a lot of variation within the hybrid families. Hybrid families showed average canker lengths between *C. mollissima* and *C. dentata*, as expected. The ANOVA revealed significant differences among family canker lengths, indicating the SSA's ability to differentiate blight resistance levels within hybrid families. However, high standard deviations relative to means suggested substantial random errors, impacting SSA results. OALDS confirms that blight tolerance and OA tolerance are polygenic traits, causing difficulties in the breeding process (Westbrook et al., 2019). However, neither the SSA nor OALDS could show statistical significance between the hybrids. Both might not be optimal for making these selections due to inherent limitations in accuracy.

**Heritability of Blight Resistance**

The Asian species, *C. henryi* and *C. seguinii*, do not appear to have resistance that is significantly greater than American chestnut in this dataset (TACF, n.d.). The result that was surprising and contradicts the paradigm that the Asian species represent sources of genetic resistance to *C. parasitica* (TACF, n.d). There was a continuous variation in the apparent resistance of species. The North American chinquapins have intermediate resistance between *C. dentata* and *C. mollissima*. *C. sativa* was second to *C. mollissima* for most blight resistant which is atypical of the species (Figure 4).

**Limitations**

My experiment had atypical results from *C. henryi*. We expected *C. henryi* to be very resistant to *C. parasitica*; however, in this experiment *C. henryi* was very susceptible to *C. parasitica*. This could be the result of several factors. The seeds were planted over a period of a month, so some plants had more growing time than others. The *C. henryi* could have been at a disadvantage from the beginning. Then when the plants were moved outside *C. henryi* were outcompeted by the larger species. It is also important to note that the Fortwood Greenhouse was designed to allow *C. dentata* the best growing conditions, which could also cause a disadvantage to *C. henryi*. *C. parasitica* may have taken advantage of these disadvantages causes the atypical result in my experiment.

*C. sativa* shows more resistance in my experiment than it does in Nugyen 2023 in the same growing conditions. The *C. sativa* seeds for this year's SSA were purchased from a commercial grocery store with the label "grown in Italy." We need to be cautious of these results because we only have two sources, one from the supermarket and the other from a mail order. We did not grow the parent trees or have close contacts to those who did grow the parent plant for the *C. sativa* sample. It is possible that the trees marked as *C. sativa* could have been a *C. sativa* x *C. crenata* hybrid due to its elevated level of resistance. I think this is unlikely because the *C. sativa* plants look and grew with typical *C. sativa* characteristics. It is more probable that our *C. sativa* sample liked the conditions in the greenhouse and nursery, producing the atypical results.

In this experiment, it was difficult to measure the orange zones due to inconsistencies in defining the endpoint. The discoloration within the cankers varied from pale white, light brown, and orange. This made it difficult to determine the length of the orange zone and resulted in high

standard errors and outliers in the measurements. To address this issue in future experiments, I recommend establishing a standardized color scale for defining the orange zone and employing multiple trained individuals to cross-verify measurements. Although I opted to conduct the measurements alone to minimize errors stemming from differing interpretations by multiple volunteers, random errors persisted, emphasizing the importance of having more than one person verify each measurement for accuracy.

In the fall of 2024, another student will continue both the small stem assay and the oxalic acid leaf disk assay on a larger sample of trees. Further investigations will continue to explore correlations between these two screening methods.

Increasing sample sizes (especially in OALDS) may lead to more significant results, while adjustments in the number of disks used and consideration of different plant parts could affect outcomes. However, given the novelty of this method, it should be evaluated alongside the small stem assay for comparison.

### Future Studies

Increasing sample sizes (especially in OALDS) may lead to more significant results, while adjustments in the number of disks used and consideration of different plant parts could affect outcomes. However, given the novelty of this method, it should be evaluated alongside the small stem assay for comparison.

## Conclusion

SSA and OALDs are beneficial in conjunction with the standard screening methods. Neither the SSA and nor OALDS should be solely relied upon for making artificial selections for blight resistance within families. The SSA can validate the accuracy of parental selections for

each family. All surviving trees from the experiment will be transplanted to an experimental orchard in Middle Tennessee and monitored for their survivability.

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